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Applicant(s): FOX et al.

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Title: **MODIFIED ACYL CARRIER PROTEINS****COMBINED RULE 131/132 DECLARATION OF INVENTORS**  
**BRIAN G. FOX AND JEFFREY A. HAAS**

Commissioner for Patents  
P.O. Box 1450  
Alexandria, VA 22313-1450

To the Commissioner:

Your Declarants, Brian G. Fox and Jeffrey A. Haas, individually and collectively,  
do hereby declare and state as follows:

1. We are each co-inventors of Claims 1-30 of the above-identified patent application as originally filed and are co-inventors of the subject matter described and claimed therein. As such, we are, individually and collectively, intimately familiar with the above-identified patent application and the subject matter claimed therein.

2. We are each co-authors of the Haas et al. paper entitled "Chemical and Posttranslational Modification of *Escherichia coli* Acyl Carrier Protein for Preparation of Dansyl-Acyl Carrier Proteins," published in the journal *Protein Expression and Purification*, volume 20, issue 2, pages 274-284, dated November 2000, and cited as a prior publication in the above-identified patent application. We are, individually and collectively, intimately familiar with the contents of this paper.


3. The above-identified application for patent was filed on October 22, 2001, a date which is less than one year after the publication of the Haas et al. paper cited in paragraph 2.

4. Prior to November 1, 2000, having earlier jointly conceived the idea of labeling acyl carrier proteins with non-radioactive labels, we collectively began work on conjugating various non-radioactive labels, such as fluorophores, to acyl carrier proteins. In due course, we perfected the required chemistry, wrote a paper describing the same, and submitted the paper to the journal *Protein Expression and Purification* for possible publication. The paper we wrote and submitted is entitled "Chemical and Posttranslational Modification of *Escherichia coli* Acyl Carrier Protein for Preparation of Dansyl-Acyl Carrier Proteins," and is the same paper that was ultimately published (under the same title) as the applied reference discussed in paragraph 2, above. Attached hereto as Exhibit A (and incorporated herein) is a true copy of the original paper that we, Brian G. Fox and Jeffrey A. Haas, submitted to *Protein Expression and Purification* for possible publication.

5. We, Brian G. Fox and Jeffrey A. Haas, hereby declare that the document attached hereto as Exhibit A was prepared, by us, prior to November 1, 2000.

6. We, Brian G. Fox and Jeffrey A. Haas, individually and collectively, hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this Rule 131/132 Declaration is directed.

  
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Brian G. Fox

  
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Date

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Jeffrey A. Haas

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Date

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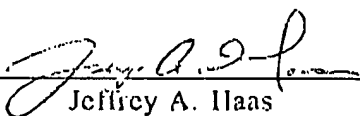
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Brian G. Fox

Date

  
Jeffrey A. Haas

6/15/04

Date

# Chemical and Posttranslational Modification of *Escherichia coli* Acyl Carrier Protein for Preparation of Dansyl-Acyl Carrier Proteins<sup>1</sup>

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## TITLE FOOTNOTE:

<sup>1</sup> This work was supported by Grant GM-50853 from the National Institutes of Health.

## RUNNING TITLE:

Dansylation of aminotyrosyl-ACP

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<sup>4</sup> Abbreviations used: ACP, acyl carrier protein; apo-ACP, form of ACP lacking phosphopantetheine; nitroTyr-ACP, form of ACP chemically modified at C<sup>ε</sup> of Tyr71 to 3-nitroTyr-ACP; aminoTyr-ACP, 3-aminoTyr-ACP; dansylaminoTyr-ACP, 3-aminoTyr-ACP modified to contain a dansyl group on the amino group; holo-ACP, biologically active form of ACP containing phosphopantetheine; ACPS, holo-ACP synthase; AAS, acyl-ACP synthetase; 18:0-ACP, stearoyl-ACP; 18:1-ACP, oleoyl-ACP; GC-MS, tandem gas chromatography and mass spectrometry; ESI-MS, electrospray ionization mass spectrometry;  $\Delta^9$ D, 18:0-ACP  $\Delta^9$  desaturase; Fd, *Anabaena* 7120 vegetative [2Fe-2S] ferredoxin; FdR, *Zea mays* NADPH:ferredoxin oxidoreductase; OD<sub>600</sub>, optical density at 600 nm; PCR, polymerase chain reaction.

**ABSTRACT:** *Escherichia coli* acyl carrier protein (ACP) contains a single tyrosine residue at position 71. The combined *o*-nitration of apo-ACP Y71 by tetranitromethane and reduction to 3-aminotyrosyl-apo-ACP were performed to introduce a specific site for attachment of a dansyl-fluorescent label. Conditions for purification and characterization of dansylaminotyrosyl-apo-ACP are reported. Dansylaminotyrosyl-apo-ACP was enzymatically phosphopantetheinylated and acylated *in vitro* with an overall ~30% yield of purified stearyl-dansylaminotyrosyl-ACP starting from unmodified apo-ACP. The steady-state kinetic parameters  $k_{\text{cat}} = 22 \text{ min}^{-1}$  and  $K_M = 2.7 \text{ }\mu\text{M}$  were determined for reaction of stearyl-dansylaminotyrosyl-ACP with stearyl-ACP  $\Delta^9$  desaturase. These results show that dansylaminotyrosyl-ACP will function well for studying binding interactions with the  $\Delta^9$  desaturase and suggest similar possibilities for other ACP-dependent enzymes. The efficient *in vivo* phosphopantetheinylation of *E. coli* apo-ACP by co-expression with holo-ACP synthase in *E. coli* BL21(DE3) using fructose as the carbon source is also reported.

Acyl carrier proteins (ACP)<sup>4</sup> are small (~8-10 kDa) acidic proteins that contain a 4'-phosphopantetheine prosthetic group. This prosthetic group is attached to a conserved serine residue in apo-ACP by holo-ACP synthase [ACPS, (1)]. In holo-ACP, the prosthetic group provides a free thiol group that is required for ACP function in a variety of biosynthetic pathways including *de novo* biosynthesis of fatty acids (1), depsipeptides (2), peptides (3), polyketides (4), and the posttranslational acylation of proteins (5).

Acyl-ACPs are also substrates for the soluble desaturases found in the plastid organelles of plants and photoauxotrophic *Euglena* (6). The stearyl-ACP  $\Delta^9$  desaturase ( $\Delta 9D$ ) from *Ricinus communis* is the best characterized member of this enzyme family (7).  $\Delta 9D$  catalyzes the NADPH- and O<sub>2</sub>-dependent insertion of a *cis*-double bond at the C9 position of 18:0-ACP to form 18:1-ACP. Non-heme diiron centers found in each subunit of the homodimeric  $\Delta 9D$  (8-10) are utilized for the O<sub>2</sub> activation steps of catalysis.

Recent studies have revealed the importance of interactions between acyl-ACP and  $\Delta 9D$  in the catalytic selectivity (11), perturbation of the ligation environment of the diiron center (12), and the accumulation of a quasi-stable peroxodiiron(III) species (13, 14). Further biophysical characterization of the protein-protein interactions involved in these catalytic phenomena could be facilitated by the availability of selective probes for complex formation. Since neither acyl-ACP nor  $\Delta 9D$  exhibit chromophoric features suitable for these studies, we have undertaken the synthesis of a suitably derivatized form of acyl-ACP. These efforts provide substrate-level quantities of ACPs with a site-specific dansyl modification at the unique Y71 of ACP (Fig. 1) for use as a fluorescent probe, and with a fatty acyl chain required for desaturase catalysis.

## **MATERIALS AND METHODS**

### *Chemicals, Enzymes, Plasmids, and Bacterial Strains*

Restriction endonucleases, T4 DNA ligase, calf intestinal phosphatase, and Deep Vent Polymerase were purchased from New England BioLabs (Beverly, MA). Coenzyme A and pantothenic acid hemicalcium salt were obtained from Sigma (St. Louis, MO). Nucleotide

triphosphates were purchased from Pharmacia Biotech (Piscataway, NJ). Oligonucleotide primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). AAS, ACPS,  $\Delta 9D$ , FdR, and Fd were expressed, purified, and characterized as previously described (15). Tetranitromethane and stearic acid were purchased from Aldrich (Milwaukee, WI). Dansyl chloride was purchased from Molecular Probes (Eugene, OR). The *E. coli* strain DH5 $\alpha$  [*supE44* $\Delta$ *lacU169*( $\phi$ 80 *lacZ* $\Delta$ M15)*hsdR17recA1 endA1 gyrA96 thi-1 relA1*] was used for general cloning steps. The *E. coli* strain BL21(DE3) [*F*<sup>-</sup> *ompT hsdS<sub>B</sub>* (*r<sub>B</sub>-m<sub>B</sub>*-) *gal dcm* (DE3)] was used as the expression host.

### *Plasmid Construction*

The isolation of plasmid DNA and all other cloning manipulations were carried out according to established procedures (16). Purified *E. coli* K12 genomic DNA was used as template for PCR amplification of the *E. coli acpP* gene. The forward primer incorporated an *NdeI* restriction site at the start codon: 5' ccgcaCATATGagcaccatcgaagaacgtgtg. The reverse primer incorporated a *XhoI* site after the stop codon: 5' caataCTCGAGttacgcctggtggccgttgatg. The resulting ~300 bp amplified fragment was purified by gel electrophoresis and extracted using the QIAEX II extraction kit (Qiagen, Valencia, CA). The purified PCR product was blunt-end ligated into the *EcoRV* site of pZero-2 (Invitrogen, Carlsbad, CA) using T4 DNA ligase. The ligation reaction was used to transform CaCl<sub>2</sub> competent *E. coli* DH5 $\alpha$  by heat shock. The transformation mixture was plated onto Luria-Bertani agar plates containing 50  $\mu$ g  $\times$  mL<sup>-1</sup> kanamycin. Plasmids were isolated from kanamycin-resistant transformants by Mini-Prep (Promega, Madison, WI) and screened for the correct insert by restriction mapping. The sequence of the amplified *acpP* gene was verified by cycle sequencing using AmpliTaq DNA polymerase, FS (Perkin-Elmer, Culver City, CA) and dye-labeled terminators at the University of Wisconsin Biotechnology Center. The *acpP* gene was removed from the pZero-2 vector by double digestion with *NdeI* and *XhoI* and ligated into similarly digested and gel-purified pET17(b) (Novagen, Madison, WI). The ligation mixture was used to transform competent *E. coli* DH5 $\alpha$  and plated onto Luria-Bertani agar plates containing 100  $\mu$ g  $\times$  mL<sup>-1</sup> ampicillin.



Plasmids were isolated and characterized as described above, and a correctly constructed plasmid was named pEACP-2 (Fig. 2A). The *acpP* gene was also subcloned from pEACP-2 by double digestion with *Xba*I and *Xho*I and ligated into similarly digested pET28(a) (encoding kanamycin resistance). This new vector was called pBHF-5 (Fig. 2B).

A coexpression vector containing the *E. coli acpP* and *acpS* genes was constructed as previously described for the coexpression vector containing the spinach *acpP* and *E. coli acpS* genes (15). This vector was named pBHF-1 (Fig. 2C).

#### *Media and Fermentation Protocols*

Fermentations were done in a 10-L New Brunswick Scientific BIOFLO 3000 bench-top fermenter (New Brunswick, NJ). The pH was maintained at 7.1 by the controlled addition of 4 M  $\text{NH}_4\text{OH}$  and 4 M  $\text{H}_2\text{SO}_4$ . The dissolved  $\text{O}_2$  level was maintained at 30% of air saturation or greater by variation of the agitation rate. Foaming was suppressed by manual addition of antifoam (Mazu DF 204, PPG Industries, Gurnee, IL). Kanamycin was not added to the culture medium in the fermenter. The cells were harvested by centrifugation at 4400g for 15 min in a Beckman J-6B centrifuge equipped with a JS-5.2 rotor (Beckman, Fullerton, CA).

For batch fermentations in Luria Bertani medium, *E. coli* BL21(DE3) was transformed with pBHF-1 or pBHF-5 and plated onto Luria Bertani agar plates containing  $50 \mu\text{g} \times \text{mL}^{-1}$  kanamycin. After 16 h, a single colony was aseptically transferred into a sterile test tube containing 5 mL of Luria Bertani medium and  $50 \mu\text{g} \times \text{mL}^{-1}$  kanamycin. The culture was grown with shaking at 37°C until the  $\text{OD}_{600}$  reached ~0.8; 50  $\mu\text{L}$  of this culture was then used to inoculate each of two 2-L flasks containing 500 mL of Luria Bertani medium and  $50 \mu\text{g} \times \text{mL}^{-1}$  kanamycin. The two 500 mL cultures were grown at 37°C until the  $\text{OD}_{600}$  reached ~1; 1 liter was then used to inoculate the fermenter containing 9 liters of Luria Bertani medium. The fermenter culture was grown at 37°C until the  $\text{OD}_{600}$  reached ~3. At this point, the culture was induced by the batch addition of filter-sterilized solutions of  $\beta$ -D-lactose (0.8% w/v), Casamino acids (0.2% w/v, Difco, Detroit, MI), and  $0.05 \text{ g} \times \text{L}^{-1}$  pantothenic acid hemicalcium salt. The induced culture was grown for 4 h, and yielded  $\sim 7 \text{ g} \times \text{L}^{-1}$  of wet cell paste.

For batch fermentations in minimal medium supplemented with Casamino acids ( $0.2 \text{ g} \times \text{L}^{-1}$ ) (16) and using fructose as the carbon source, *E. coli* BL21(DE3) was transformed with pBHF-1 and starting inocula were prepared as described above except that the 2-L flasks contained 500 mL of minimal medium with  $4 \text{ g} \times \text{L}^{-1}$  of D-fructose,  $2 \text{ g} \times \text{L}^{-1}$  Casamino acids, and  $50 \mu\text{g} \times \text{mL}^{-1}$  kanamycin. When the 500 mL cultures reached an  $\text{OD}_{600}$  of  $\sim 1$ , they were used to inoculate a fermenter containing 9 liters of the same medium prepared without kanamycin. The culture grew to  $\text{OD}_{600} \sim 7$  before all of the fructose was consumed as indicated by a sudden increase in the dissolved  $\text{O}_2$  concentration and corresponding drop in the agitation rate (17). At this point, the cells were induced by the addition of lactose as described above, and the temperature was adjusted to  $30^\circ\text{C}$ . The induced culture was grown for 4-5 h, and yielded  $\sim 12 \text{ g} \times \text{L}^{-1}$  of wet cell paste.

For batch fermentations in minimal medium supplemented with Casamino acids ( $0.2 \text{ g} \times \text{L}^{-1}$ ) and containing  $6 \text{ g} \times \text{L}^{-1}$  glucose, the procedures described above for fructose growth were used. The  $6 \text{ g} \times \text{L}^{-1}$  of glucose was depleted when the culture reached an  $\text{OD}_{600} \sim 5$ -6, and induction, protein expression, and cell harvest were as described above. A yield of  $\sim 11 \text{ g} \times \text{L}^{-1}$  of wet cell paste was obtained.

#### *ACP Purification*

All purification steps were performed at  $4^\circ\text{C}$ . A 50 g block of frozen cell paste was broken into pieces and resuspended in 100 mL of 100 mM Tris, pH 8.0 in a stainless steel beaker. Lysozyme, DNase, and RNase (0.15 mg of each, Sigma) were added to the suspension. The cell mixture was sonicated for a total of 6 min using 30 s pulses (Fisher Model 550 Sonic Dismembrator, 3/4 inch disruptor horn, 100% of maximum output). During sonication, the temperature of the cell suspension was maintained below  $7^\circ\text{C}$  by placing the beaker in an ice bath containing a saturated NaCl solution. The sonicated cell suspension was centrifuged at  $39,000g$  for 1 h to remove cell debris. The supernatant was diluted 2-fold with 25 mM MES, pH 6.1 and loaded onto a Fast Flow DEAE-Sepharose CL-6B (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) column ( $48 \times 250 \text{ mm}$ ) equilibrated in 25 mM MES, pH 6.1. The column

was washed with 0.75 L of 25 mM MES, pH 6.1. The protein was eluted in a 1.6 L linear gradient from 0 to 0.85 M NaCl in 25 mM MES, pH 6.1, at a linear flow rate of  $3 \text{ cm} \times \text{h}^{-1}$ . Fractions were analyzed by SDS-PAGE and peak fractions were pooled and concentrated by ultrafiltration (YM3 membrane, AMICON, Inc., Beverly, MA). The concentrated peak fractions were loaded onto a Sephacryl S-100 column ( $48 \times 1000 \text{ mm}$ ) equilibrated with 25 mM MES, pH 6.1, containing 0.1 M NaCl and eluted at  $6.6 \text{ cm} \times \text{h}^{-1}$ . Peak fractions were analyzed by SDS-PAGE, pooled and concentrated by ultrafiltration.

#### *Nitration of Tyr71 of apo-ACP with Tetranitromethane*

The nitration reaction mixture typically contained 240  $\mu\text{M}$  apo-ACP (50 mg) in 50 mM Tris, pH 8.1. Tetranitromethane was added from a 0.84 M solution prepared in 95% ethanol to give a final concentration in the reaction mixture of 50 mM. The reaction mixture was stirred for ~2.5 h at 30°C and stopped by precipitation of ACP with dilute acetic acid. NitroTyr-ACP was purified by gel filtration chromatography on a 26/10 HiPrep Sephacryl S-100 column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) in 25 mM MES, pH 6.1, at a linear flow rate of  $11 \text{ cm} \times \text{h}^{-1}$ . Fractions containing pure nitroTyr-ACP were pooled based on examination of native-PAGE gels.

#### *Reduction of NitroTyr-apo-ACP to AminoTyr-apo-ACP*

Pooled nitroTyr-ACP fractions were adjusted to pH 8.0 by the addition of 0.32 M Tris, pH 8.0 to give a final concentration of 50 mM Tris. The sample was then made anaerobic by repeated evacuation and flush with  $\text{O}_2$ -free Ar. The anaerobic sample of nitroTyr-ACP was reduced to aminoTyr-ACP by the addition of ~800  $\mu\text{L}$  of  $25 \text{ mg} \times \text{mL}^{-1}$  sodium dithionite prepared in  $\text{O}_2$ -free 1 M potassium phosphate buffer, pH 7.0. The aminoTyr-ACP was exchanged into 50 mM sodium acetate buffer, pH 4.75, by repeated concentration and dilution using ultrafiltration.

#### *Dansylation of AminoTyr-apo-ACP*

The dansylation reaction mixture contained 40  $\mu\text{M}$  aminoTyr-ACP (~35-40 mg) in 50 mM sodium acetate, pH 5.0, containing 35% (v/v) acetonitrile. Dansyl chloride was prepared

as a 15 mM dansyl chloride solution in acetonitrile. This solution was slowly added to the rapidly stirred reaction mixture to give a final concentration of 1.5 mM dansyl chloride. The reaction mixture was stirred at room temperature in the dark for 1 h, diluted 10-fold with 25 mM MES, pH 6.1, and loaded onto a 15 mL Fast Flow DEAE-Sepharose CL-6B column. The column was washed with 150 mL of 25 mM MES, pH 6.1, to remove acetonitrile and the dansyl-ACP was batch-eluted in the same buffer containing 0.75 M NaCl. This step also separated ACP from unreacted dansyl chloride, which formed a visible yellow precipitate on the column. The dansyl chloride was later removed by washing the column with 25 mM MES, pH 6.1, containing 80% (v/v) 2-propanol. The dansyl-ACP was precipitated by addition of dilute acetic acid to give a pH of ~3.9, and recovered by centrifugation. The resulting pellet was resuspended in 20 mL of 0.3 M MES, pH 6.1. DansylaminoTyr-ACP was purified from a didansyl-ACP species using a 16/10 HiLoad Q Sepharose Fast Flow column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The column was loaded with dansyl-ACP at a linear flow rate of  $15 \text{ cm} \times \text{h}^{-1}$  in 25 mM succinic acid, pH 5.0, washed with 100 mL of the 25 mM succinic acid, pH 5.0, and then washed with 100 mL of 25 mM succinic acid, pH 5.0, containing 0.05 M NaCl. The protein was eluted at a linear flow rate of  $15 \text{ cm} \times \text{h}^{-1}$  in a 300 mL linear gradient from 0.05 to 0.25 M NaCl in 25 mM succinic acid, pH 5.0. Fractions containing dansylaminoTyr-ACP or didansyl-ACP were identified by native-PAGE. Both dansylaminoTyr-ACP and didansyl-ACP could be detected by fluorescence on a light box under reflected 300 nm light (prior to Coomassie staining) or by Coomassie staining.

#### *In Vitro Phosphopantetheinylation and Acylation of ACP*

Phosphopantetheinylation reactions typically contained ~40  $\mu\text{M}$  dansylaminoTyr-ACP (~20 mg), 100  $\mu\text{M}$  coenzyme A, 10 mM  $\text{MgCl}_2$ , and 1  $\mu\text{M}$  ACPS in 50 mM Tris, pH 8.8. The reaction was incubated with stirring at 25°C for 1 h, and terminated by addition of the acylation buffer to bring the final solution to 15  $\mu\text{M}$  ACP, 0.4 M LiCl, 10 mM  $\text{MgCl}_2$ , ~2-3% Triton X-100, 5 mM ATP, 3 mM DTT, and 260  $\mu\text{M}$  stearic acid (delivered as 70  $\mu\text{mol}$  stearic acid dissolved in 1 mL toluene) in 50 mM Tris, pH 8.0. AAS (2.5 mg) was added and the

reaction was stirred for ~16 h at 25°C. To purify acyl-ACP from the other reaction components, the acylation reaction was diluted 10-fold with 25 mM MES, pH 6.1, and loaded onto a 15 mL Fast Flow DEAE Sepharose CL-6B column. The column was successively washed with 150 mL of 25 mM MES, pH 6.1, 100 mL of an 80:20 (v/v) mixture of 2-propanol and 25 mM MES, pH 6.1, and 150 mL of 25 mM MES, pH 6.1. The purified acyl-ACP was eluted in 40 mL of 25 mM MES, pH 6.1, containing 0.75 M NaCl, and the eluate was adjusted to a pH of ~3.9 with dilute acetic acid to precipitate the acyl-ACP. The precipitated acyl-ACP was collected by centrifugation and resuspended in 0.1 M MES, pH 6.5.

### *Electrophoresis Methods*

Protein expression was detected by denaturing gel electrophoresis (70 × 80 × 0.75 mm gels, Bio-Rad, Hercules, CA) using 10% Tris-Tricine polyacrylamide gels and Coomassie Blue staining (18). Protein standards were obtained from Novex (San Diego, CA).

Chemical modification of apo-ACP Y71 was detected by native gel electrophoresis (70 × 80 × 0.75 mm gels). Continuous gels consisting of 13% acrylamide, 0.5% *N,N'*-methylenebisacrylamide, 43 mM imidazole, 35 mM HEPES, pH 7.4, and 1 M urea were used for detection of nitroTyr-ACP. A buffer containing 43 mM imidazole and 35 mM HEPES, pH 7.4 was used for both anode and cathode buffers. For detection of dansylaminoTyr-ACP, the same buffer system described for detection of nitroTyr-ACP was used with 16% acrylamide and 0.7% *N,N'*-methylenebisacrylamide gels.

### *Purification of Acyl-ACP by Preparative Native-PAGE*

For preparative native gel electrophoresis, a gel solution containing 13% acrylamide, 0.5% *N,N'*-methylenebisacrylamide, 0.37 M Tris, pH 9.0, and 1 M urea was degassed immediately prior to casting the gel in the Model 491 PrepCell (BioRad, Hercules, CA). A 190 mM glycine, 25 mM Tris, electrophoresis/elution buffer was degassed by vacuum filtration immediately prior to electrophoresis. The pH of this buffer was not adjusted. The fractions from the PrepCell eluate were analyzed with electrophoresis 70 × 80 × 0.75 mm slab gels using the same gel system as used in the PrepCell.

Preparative-scale native-PAGE was carried out using a Model 491 Prep Cell (Bio-Rad, CA). A 37 mm diameter gel assembly tube was used to cast 3.5 or 5 cm length gels at 4°C. A typical sample contained 8-10 µmol of acyl-ACP in ~3 mL of 0.1 M MES, pH 6.5, and was mixed with 1 mL of sample loading buffer [0.1 M Tris, pH 6.8, 30% (w/v) glycerol, and 0.03% (w/v) bromphenol blue] immediately prior to electrophoresis. The apparatus was used following the manufacturer's instructions at 7 W constant power. Peak fractions were pooled based on assay by UV detection and by examination of analytical native-PAGE gels. Pooled peak fractions were concentrated by precipitation with dilute acetic acid and the resuspended protein was separated from bromphenol blue using a G-25/150 gel filtration column (25 × 75 mm) equilibrated in 25 mM MES, pH 6.5. The purified acyl-ACP was concentrated by precipitation with dilute acetic acid and quantitated as described below.

#### *Quantitation of Acyl-ACPs*

Acyl-ACPs were quantitated by hydrolysis of the thioester linkage followed by colorimetric determination of the free thiol present in holo-ACP using 5,5'-dithio-bis-(2-nitrobenzoic) acid (DTNB). The reaction mixture consisted of ~15-50 µM acyl-ACP in 200 µL of 60 mM NaOH (from a certified 0.25 N NaOH stock solution; Fisher, Springfield, NJ) and was incubated at 40°C for 10 min. The cleavage reaction was stopped and the reaction mixture was adjusted to neutral pH by addition of 50 µL of 0.25 M HCl (certified 0.25 N HCl stock solution; LabChem, Inc., Springfield, NJ). The DTNB assay was performed by addition of 230 µL of terminated acyl-ACP cleavage reaction to 700 µL of 0.2 mM DTNB in 50 mM phosphate buffer, pH 7.0 in a quartz cuvette. The amount of thionitrobenzoate formed by reaction of holo-ACP with DTNB was determined using optical spectroscopy and a molar absorptivity of  $13,600 \text{ M}^{-1} \times \text{cm}^{-1}$  at 412 nm (19). A control reaction where deionized water was substituted for both NaOH and HCl was used to estimate the background amount of holo-ACP.

## *Electrospray Ionization Mass Spectrometry*

Protein mass spectra were obtained on a Perkin-Elmer-Sciex API 365 triple quadrupole electrospray ionization mass spectrometer at the Mass Spectrometry Facility of the University of Wisconsin Biotechnology Center. Samples were equilibrated with 20 mM ammonium acetate, pH 6.1, using a HiPrep 26/60 Sephacryl S-100 column (Pharmacia) at a flow rate of 1 mL min<sup>-1</sup> to remove sodium. Alternatively, a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL) was used to dialyze a 300 µL sample against three 1-liter changes of ammonium acetate buffer.

## *Stearoyl-ACP $\Delta^9$ Desaturase Assay*

In a typical assay, 1-50 nmol of 18:0-dansylaminoTyr-ACP, 0.2 nmol of FdR, 1 nmol of Fd, 0.02 nmol of  $\Delta^9$ D, and 1700 nmol of NADPH were placed in 1 mL of 50 mM HEPES, pH 7.8, containing 50 mM NaCl in an open 5 mL autosampler vial. Reaction vials were shaken at 100 rpm in a 25°C water bath. The reactions were started by the addition of  $\Delta^9$ D and at timed intervals 200 µL aliquots were withdrawn and quenched by addition to 150 µL of tetrahydrofuran. Steady-state kinetic analysis was performed as previously described (11). The fatty acyl-chains were reductively cleaved from ACP, extracted, derivatized, and quantitated by GC-MS as previously described (11). The  $k_{cat}$ -values are reported with respect to diiron center concentration (11).

## **RESULTS**

### *Expression and In Vivo Modification of Recombinant ACP*

The vector pEACP-2 (Fig. 2A) has a T7 promoter and does not constitutively express *lacI*<sup>Q</sup>, resulting in uncontrolled basal expression. Consequently, liquid cultures inoculated with pEACP-2 transformants did not reach observable densities (Table 1). However, when *acpP* was cloned into a vector containing the T7lac promoter and the *lacI*<sup>Q</sup> gene for constitutive expression of lac repressor (pBHF-5, Fig. 2B), cells were capable of growth and expression of ~105 mg × L<sup>-1</sup> ACP (Table 1). During these fermentations, the culture density reached a maximum ~2 h after induction with lactose at 37°C, and then the culture density began to

rapidly decline. By decreasing the temperature to 30°C at induction, the growth period could be extended to ~4 h, but this change did not increase the yield of purified ACP (data not shown). The majority of ACP expressed from pBHF-5 cells was in the apo-form (Table 1, >95%). ESI-MS also revealed that the purified apo-ACP contained a fraction (~15%) with mass corresponding to an additional 131 Da, which likely corresponded to incomplete removal of the *N*-terminal Met following translation (15, 20).

Coexpression of ACPs from either spinach or *Streptomyces* with *E. coli* ACPS gave predominantly holo-ACP. Fig. 2C shows a similarly constructed bicistronic vector containing both *E. coli* *acpP* and *acpS* under control of the T7lac promoter. While pBHF-1 transformants grown in minimal medium containing glucose as the carbon source did not express either ACP or ACPS, ~15 mg × L<sup>-1</sup> of ACP was obtained from coexpression in Luria Bertani medium, with ~50-75% of the recovered ACP in the holo-ACP form (Table 1).

When pBHF-1 transformants were grown in minimal medium containing fructose as the carbon source, approximately the same amount of ACP was recovered (14 mg × L<sup>-1</sup>, Table 1). However, in the fructose medium, essentially complete posttranslational phosphopantetheinylation was obtained (>95%, Table 1).

#### *Nitration of Tyr71 of Apo-ACP*

Apo-ACP was used as a substrate for the nitration reaction in order to eliminate the requirement to chemically protect the unique, free thiol group found in holo-ACP. The nitration reaction gives substitution at the C<sup>ε</sup> position of Tyr (21). A mixture of mono- and dinitro-ACP species was obtained from this reaction. These species were resolved by native-PAGE and gel filtration chromatography, and were further characterized by ESI-MS (data not shown). NitroTyr-ACP was purified in ~80% yield (Table 3) by gel filtration chromatography. Fig. 3 shows the absorbance spectrum and ESI-MS spectrum of purified nitroTyr-ACP. NitroTyr-ACP had a pH dependent absorption spectrum (Fig. 3A) that was nearly identical to authentic 3-nitrotyrosine and also displayed an isosbestic point at 381 nm (21). The ESI mass spectrum (Fig. 3B, Table 2) contained two peaks corresponding to the calculated molecular



weight of nitroTyr-ACP without *N*-terminal Met (8555 Da) and nitroTyr-ACP with *N*-terminal Met (8686 Da).

#### *Reduction of NitroTyr-ACP to AminoTyr-ACP*

NitroTyr-ACP was reduced to aminoTyr-ACP by sodium dithionite. In anaerobic samples, the 430 nm absorbance of nitroTyr-ACP was lost within 2 min after the addition of sodium dithionite, and a new absorbance feature was observed at 275 nm (Fig. 4A). The optical band was also pH sensitive and the maximum shifted to ~300 nm at pH 5.5. ESI-MS of the reduction product verified the conversion of nitroTyr- to aminoTyr-ACP (Fig. 4B), and again revealed two major species corresponding to aminoTyr-ACP with and without *N*-terminal Met (8524 Da and 8655 Da, respectively) in the same percentage as the starting nitroTyr-ACP preparation.

#### *Dansylation of AminoTyr-ACP*

The dansylation of aminoTyr-ACP was performed at pH 5.0 in order to reduce the modification of other amine groups present in ACP. Under these conditions, apo-ACP was not dansylated in a control reaction after 1 h at room temperature as determined by native-PAGE and either UV illumination or Coomassie staining. The reaction of aminoTyr-ACP with dansyl chloride typically produced a mixture of mono- and didansylated ACP products (~65% and ~35% respectively, as determined by native-PAGE). DansylaminoTyr-ACP was purified from didansyl-ACP by anion exchange chromatography in succinate buffer at pH 5.0. The optical and ESI-MS spectra of purified dansylaminoTyr-ACP are shown in Fig. 5. The absorbance spectrum (Fig. 5A) was similar to that reported for dansyl-aminotyrosine model compounds (22), and exhibited absorption maxima at 290 and 325 nm. DansylaminoTyr-ACP was also detected in native-PAGE gels by UV irradiation (Fig. 6). ESI-MS revealed one predominant peak (~90%, Fig. 5B), corresponding to the predicted mass of dansylaminoTyr-ACP minus the *N*-terminal Met (8757 Da). The sample also contained small fractions of dansylaminoTyr-ACP plus the *N*-terminal Met (8889 Da) and aminoTyr-ACP (8524 Da).

#### *Phosphopantetheinylation and Acylation of DansylaminoTyr-ACP*

The *in vitro* phosphopantetheinylation of dansylaminoTyr-ACP was performed as previously reported for spinach ACP (15). Subsequent reaction with AAS and stearic acid yielded 18:0-dansylaminoTyr-ACP in greater than 95% yield as determined by denaturing gel electrophoresis (Fig. 6A lane 3 and 6B, lane 2) and by DTNB assay (data not shown). Figure 6B (lanes 1 and 2, respectively) shows the fluorescence emission from dansylaminoTyr-ACP and 18:0-dansylaminoTyr-ACP when the denaturing electrophoresis gel was illuminated with reflected UV light. The overall yield for production of 18:0-dansylaminoTyr-ACP starting from 55 mg of unmodified apo-ACP was 17 mg (31%, Table 3).

#### *Purification of Acyl-ACP by Preparative Native-PAGE*

The *in vitro* acylation of ACP using AAS and 10:0 to 18:0 fatty acids produced high yields of acyl-ACP (23). In contrast, reactions performed with unusual fatty acids gave moderate yields (~40-60%, J. Haas, J. Broadwater, B. Laundre, B. Fox unpublished results). Therefore, preparative scale native-PAGE was investigated as a method to purify acyl-ACPs from holo- and apo-ACP on a 100 mg (~12  $\mu$ mol) scale. The high pH (9.0) of the buffer system yielded an  $R_f$  value of 1 for *E. coli* acyl-ACP, and consequently, minimized the time required for electrophoresis. However, the alkaline pH contributed to the relative lability of the thioester linkage of acyl-ACP. At pH 9, recovery of acyl-ACP from 5 cm gels was 45%, while the use of a 3.5 cm gel reduced the electrophoresis time and improved the recovery of acyl-ACP to 77%. Further trials revealed that the 3.5 cm gel was the minimum length that could still provide complete resolution of acyl-ACP from holo-ACP at the desired loading. The use of a pH 7.4 buffer system, which should have given improved stability of the thioester bond, resulted in a complete loss of acyl-ACP due to a dramatic decrease in  $R_f$  and corresponding increase in electrophoresis time.

#### *Desaturation of 18:0-dansylaminoTyr-ACP*

A steady-state kinetic analysis of  $k_{cat}/K_M$  was recently reported for the reaction of  $\Delta 9D$  with 15:0- to 19:0-ACPs (11). This study revealed the importance of acyl chain length on catalytic enhancement. To evaluate the effect of the dansyl modification on catalysis by  $\Delta 9D$ ,

the steady-state kinetic parameters  $k_{\text{cat}} = 22 \pm 0.76 \text{ min}^{-1}$  and  $K_M = 2.7 \pm 0.43 \text{ }\mu\text{M}$  were determined for the  $\Delta 9\text{D}$ -catalyzed desaturation of 18:0-dansylaminoTyr-ACP (Fig. 7). These values are similar to those previously determined for 18:0-ACP ( $k_{\text{cat}} = 33 \pm 0.80 \text{ min}^{-1}$ ,  $K_M = 3.3 \pm 0.42 \text{ }\mu\text{M}$ ) (11). Furthermore, the selectivity for 18:0-dansylaminoTyr-ACP ( $k_{\text{cat}}/K_M = 8 \text{ }\mu\text{M}^{-1}\cdot\text{min}^{-1}$ ) was closer to that observed for 18:0-ACP ( $k_{\text{cat}}/K_M = 10 \text{ }\mu\text{M}^{-1}\cdot\text{min}^{-1}$ ) than for 17:0-ACP ( $k_{\text{cat}}/K_M = 2.5 \text{ }\mu\text{M}^{-1}\cdot\text{min}^{-1}$ ) (11). In addition, no change in the positional specificity for double bond insertion was observed for 17:0-ACP. These measurements demonstrate the competency of 18:0-dansylaminoTyr-ACP as a substrate for  $\Delta 9\text{D}$ .

## DISCUSSION

In this work, the efficient modification of Tyr71 in apo-ACP was described. The reported chemical modifications create a single, well defined location for placement of a fluorescent label. DansylaminoTyr-ACP was phosphopantetheinylated and acylated in high yield, and the steady-state kinetic competence of 18:0-dansylaminoTyr-ACP for desaturation by  $\Delta 9\text{D}$  was demonstrated. Taken together, these methods provide milligram quantities of purified ACP containing both acyl chains and fluorescent reporters.

### *Apparent Toxicity of apo-ACP*

The apparent toxicity of *acpP* overexpression to *E. coli* has been previously reported (24). This conclusion is supported by the lack of cell growth observed from transformants containing the poorly regulated pEACP-2 (Table 1) and the behavior of tightly regulated pBHF-5 transformants after induction. The use of pET28(a), which contains the T7lac promoter and *lacI<sup>q</sup>* (pBHF-5, Fig. 2B), gave a similar level of ACP expression (Table 1) to that obtained previously with the pET-pLysS system (20). However, the pET28-based construct does not produce lysozyme, which can lead to autocatalytic culture lysis in vigorously stirred fermenters. The yield of purified ACP obtained from coexpression with ACPS (in either Luria Bertani or minimal medium) was lower than that obtained when ACP was expressed without ACPS (Table 1). However, since the pBHF-1 coexpression vector also yielded large quantities of ACPS (~1:1 production based on examination of cell-free extracts in denaturing

electrophoresis gels), this additional protein expression may have reduced the capacity for ACP production by the host cell.

#### *Effect of Growth Medium on Posttranslational Modification of ACP*

Recently, Aristidou, *et al.* reported improved recombinant gene expression in *E. coli* when fructose was substituted for glucose as the carbon source (25). This favorable result was postulated to arise from the tighter regulation of fructose transport into cells, which resulted in a lowering of the Crabtree effect and consequent acidogenesis (25). As shown in Table 1 for pBHF-1 transformants, growth in a minimal medium containing glucose did not support the expression of either ACP or ACPS. However, when fructose was substituted for glucose, the same pBHF-1 transformants were capable of expression and efficient posttranslational modification of ACP ( $\sim 14 \text{ mg} \times \text{L}^{-1}$ , >95% holo-ACP). While these experiments do not address the molecular basis for the increased posttranslational modification, they nevertheless further suggest an expanded potential for the use of fructose as a carbon source for recombinant *E. coli* fermentations.

#### *Chemical Modification of Apo-ACP*

In order to facilitate further studies of  $\Delta 9\text{D}$  substrate selectivity, a specific site of attachment of a fluorescent probe to ACP was desired. Since *E. coli* ACP contains at least one of every polar and charged amino acid, modification strategies directed toward these amino acids were unlikely to give the desired unique labelling. Furthermore, a role for *N*-terminal residues in ACP stability has previously been reported (26). Thus labelling of the *N*-terminus was not desirable.

The *o*-nitration of tyrosine with tetranitromethane followed by reduction with sodium dithionite produces *o*-aminotyrosine, and this introduces a new amine group into the protein with a unique  $\text{pK}_a$  value of  $\sim 4.75$  (21, 27). Since the sole Tyr residue (Tyr71) of *E. coli* ACP is near the *C*-terminus (Fig. 1), and since modification of the *C*-terminus of spinach ACP did not alter reactivity with ACPS, AAS, or  $\Delta 9\text{D}$  (11, 15), Tyr71 was targeted for chemical modification and subsequent attachment of a dansyl group.

As previously reported (28), the nitration of apoTyr71-ACP required slightly higher temperature than for the same reaction with free tyrosine (21). While this suggested that the ACP polypeptide may hinder reaction of Tyr71, purified nitroTyr-ACP had a pH-dependent optical spectrum (Fig. 3A) that was nearly identical to 3-nitrotyrosine (21). NitroTyr-ACP was also readily reduced to aminoTyr-ACP under identical conditions to those reported for 3-nitrotyrosine (27), yielding the corresponding change in absorbance spectrum (Fig. 4A).

#### *Characterization of Dansylaminotyrosyl-ACP*

Dansylation of aminoTyr-ACP yielded dansylaminoTyr-ACP (~65%) and a didansylated product (~35%). Since a control reaction with apo-ACP did not yield dansylated protein, the presence of either 3-aminoTyr or dansylaminoTyr may enhance the reactivity of a remote site on ACP. DansylaminoTyr-ACP was purified from the didansyl species by anion exchange chromatography and the absorbance spectrum of dansylaminoTyr-ACP (Fig. 5A) was similar to that of dansylaminoTyr model compounds (22). DansylaminoTyr-ACP behaved indistinguishably from apo-ACP during *in vitro* phosphopantetheinylation and acylation (Fig. 6). Furthermore, the presence of dansylaminoTyr had no significant effect on the desaturation reaction (Fig. 7B), and the  $k_{\text{cat}}/K_M \sim 8 \mu\text{M}^{-1} \text{min}^{-1}$  determined for 18:0-dansylaminoTyr-ACP was similar to that of 18:0-ACP ( $k_{\text{cat}}/K_M = 10 \mu\text{M}^{-1} \text{min}^{-1}$ ) (11). Taken together, these results show that dansylation of Tyr71 does not alter the reactivities of three different enzymes that utilize various forms of ACP as a substrate. Tests of the efficacy of dansylaminoTyr-ACP as an catalytically-silent spectroscopic probe of protein-protein interactions during the desaturase reaction are now underway.

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**FIG. 1.** Ribbon diagram (30) depicting the structure of *E. coli* ACP as determined by NMR spectroscopy (31). Ser36, the site of 4'phosphopantetheine attachment and Tyr71, the site of the chemical modifications described in this paper, are also indicated.

**FIG. 2.** *Escherichia coli* ACP expression vectors. (A) map of plasmid pEACP-2 used for expression of *acpP*. (B) map of plasmid pBHF-5 used for expression of *acpP*. (C) map of plasmid pBHF-1 used for coexpression of *acpP* and *acpS*.

**FIG. 3.** Characterization of purified nitroTyr-ACP. (A) absorbance spectrum in 50 mM MES, pH 5.5 (dashed-line) or 50 mM Tris pH 8.8 (solid-line). (B) ESI-MS showing nitroTyr-ACP without (8555 Da) and with (8686 Da) *N*-terminal Met. The minor peak at 8575 Da is likely a  $\text{Ca}^{2+}$  adduct of nitroTyr-ACP.

**FIG. 4.** Characterization of purified aminoTyr-ACP. (A) absorbance spectrum in 50 mM MES, pH 5.5 (dashed-line) or 50 mM Tris pH 8.8 (solid-line). (B) ESI-MS showing aminoTyr-ACP without (8524 Da) and with (8655 Da) *N*-terminal Met. Minor peaks at 8544 and 8564 Da are likely  $\text{Ca}^{2+}$  adducts of aminoTyr-ACP. The peak at 8604 Da is probably dinitro-ACP. The ESI-MS of nitroTyr-ACP shown in Fig. 3 was from a separate trial and did not contain any dinitro-ACP.

**FIG. 5.** Characterization of purified dansylaminoTyr-ACP. (A) absorbance spectrum in 25 mM succinate, pH 5.0. (B) ESI-MS showing dansylaminoTyr-ACP (8757 Da), aminoTyr-ACP (8524 Da), and dansylaminoTyr-ACP with *N*-terminal Met (8889 Da). The peak at 8780 Da is likely a  $\text{Na}^+$  adduct of dansylaminoTyr-ACP.

**FIG. 6.** Denaturing electrophoresis gel showing dansylaminoTyr-ACP and 18:0-dansylaminoTyr-ACP. (A) Coomassie Blue-stained gel containing molecular mass standards, lane 1; dansylaminoTyr-ACP, lane 2; 18:0-dansylaminoTyr-ACP, lane 3. (B) Photograph of the gel from (A) placed on a light box under 300 nm reflected light prior to Coomassie staining; dansylaminoTyr-ACP, lane 1; 18:0-dansylaminoTyr-ACP, lane 2.

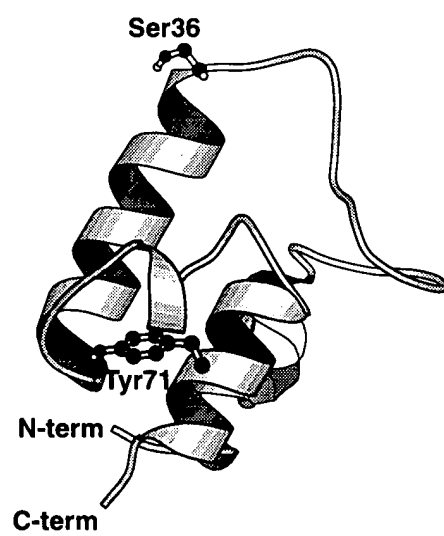
**FIG. 7.** Desaturation of 18:0-dansylaminoTyr-ACP. (A) Time-dependent accumulation of 18:1 at a fixed initial 18:0-dansylaminoTyr-ACP (2  $\mu\text{M}$ ). (■) Nanomoles of 18:1 produced. The

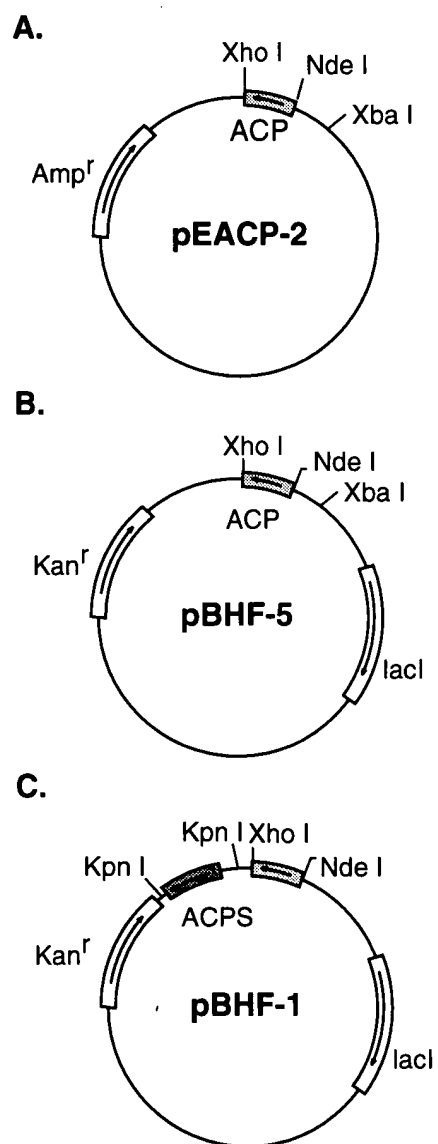
solid line is a linear least-squares fit ( $r^2 = 0.99$ ) whose slope is the initial desaturation rate,  $v_o$ .

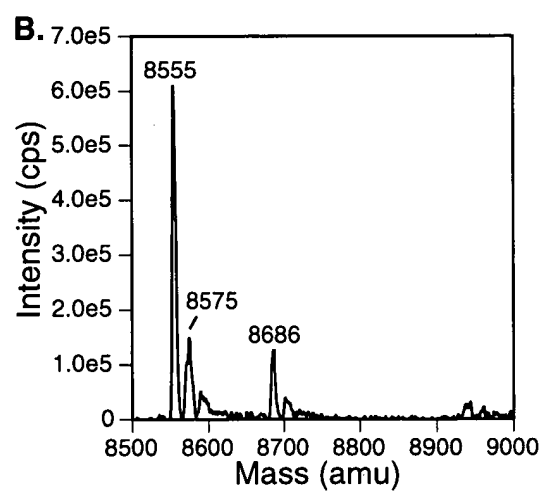
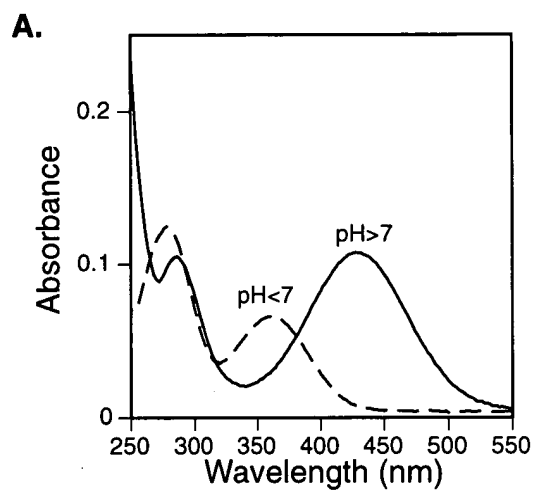
(B) Dependence of  $v_o$  as determined in (A) on the concentration of 18:0-dansylaminoTyr-ACP.

(■) Measured  $v_o$  ( $\text{min}^{-1}$ ). The solid line is a nonlinear least squares fit ( $r^2 = 0.98$ ) to the Michaelis-Menten equation,  $v_o = k_{\text{cat}} \times [S]/(K_M + [S])$ .

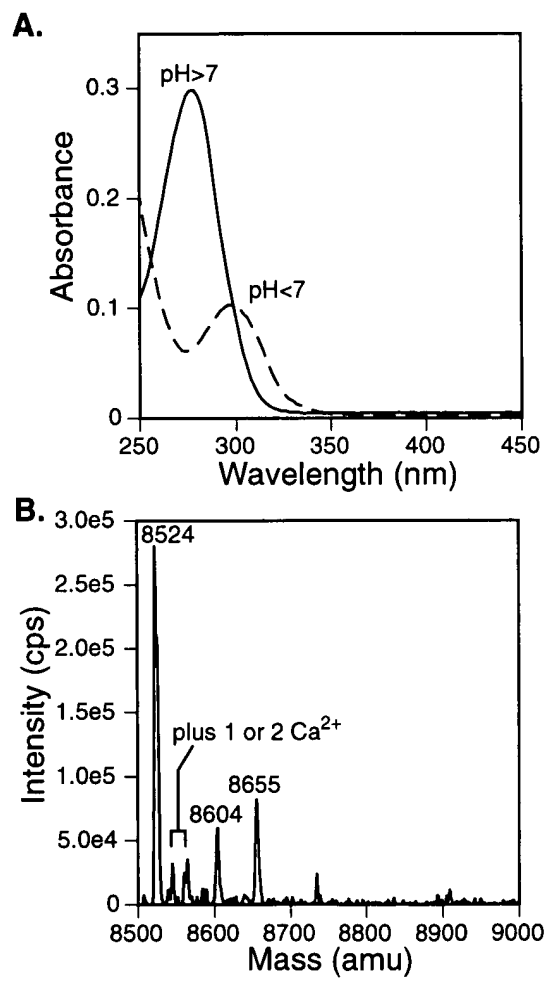
"DansylaminoTyr-ACP..." FIG.1 (TOP)



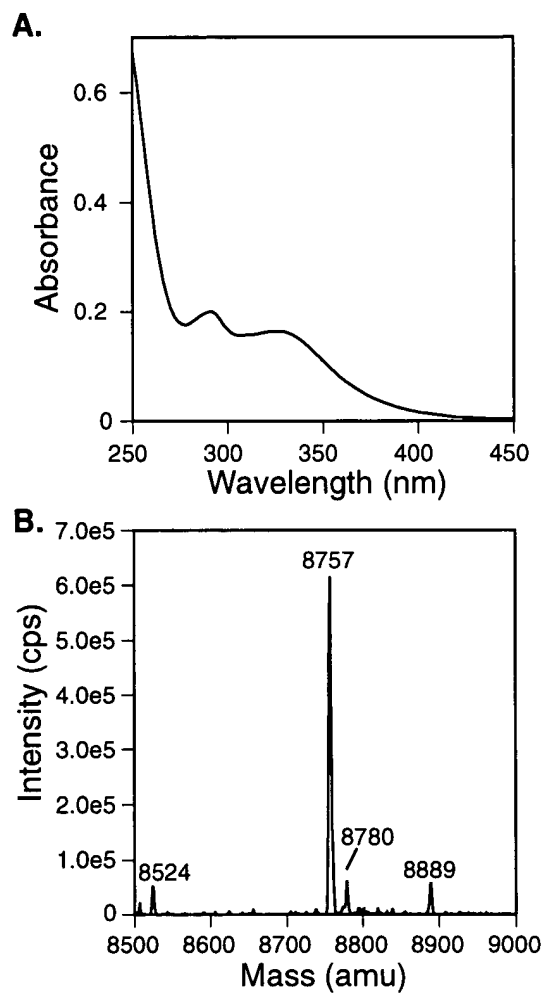




"DansylaminoTyr-ACP..." FIG.4 (TOP)



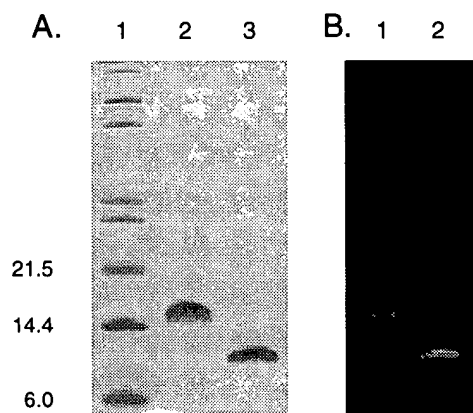
"DansylaminoTyr-ACP..." FIG.5 (TOP)





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"DansylaminoTyr-ACP..." FIG.6 (TOP)



"DansylaminoTyr-ACP..." FIG.7 (TOP)

